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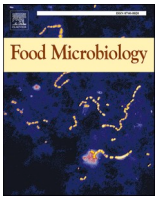

Selective pre-enrichment method to lessen time needed to recover *Salmonella* from commercial poultry processing samples

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ABSTRACT

Conventional *Salmonella* detection is time consuming, often employing a 24-h pre-enrichment step in buffered peptone water (BPW), followed by a 24-h selective enrichment in either Rappaport Vassiliadis (RV) or tetrathionate (TT) broths before streaking onto selective indicator agar. To reduce this time, we sought to optimize pre-enrichment for *Salmonella* recovery by evaluating the addition of selective chemicals to BPW. Duplicate samples each representative of 500 carcasses were collected by catching processing water drip under moving carcass shackle lines immediately after feather removal in each of nine commercial processing plants. Carcass drip samples were cultured under selective pre-enrichment conditions in parallel with BPW pre-enrichment followed by RV and TT selective enrichment. Addition of bile salts (1 g/L) and novobiocin (0.015 g/L) resulted in *Salmonella* recovery from 89% samples when plated directly after pre-enrichment compared to 67% recovery in non-selective BPW alone. *Salmonella* serovar identities were determined using CRISPR-SeroSeq. Overall, serovars matched between selective pre-enrichment and traditional enrichment methods. These data suggest that increasing the selectivity of *Salmonella* pre-enrichment step may lessen the need for a separate selective enrichment step thereby reducing time required for *Salmonella* isolation by 24 h.

1. Introduction

Salmonella is a leading bacterial cause of foodborne illnesses in the United States, causing an estimated one million illnesses each year (Scallan et al., 2011; Tack et al., 2019) and imposing an annual economic burden of approximately $3.7 billion (Hoffmann et al., 2015). Despite improvements to mitigate *Salmonella* during processing, chicken and chicken-related products remain a source of *Salmonella* outbreaks (IFSAC, 2018). In addition to regulatory surveillance by the United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS), many poultry companies perform additional *Salmonella* surveillance. Accurate and rapid surveillance for *Salmonella* is required as a key step in assessing whether mitigation strategies are effective.

The gold standard for *Salmonella* detection on broiler carcasses is a culture-based protocol that includes several steps and takes multiple days to complete (USDA, 2014). In the first non-selective pre-enrichment step, a single carcass is rinsed in 400 ml of buffered peptone water (BPW) for 1 min and then 30 ml of the rinsate is removed and added to the same volume of fresh BPW before being incubated for 24 h (United States Department of Agriculture, F.S.I.S., 1998). In the second selective-enrichment step, the pre-enriched BPW is inoculated into two selective enrichment broths such as Rappaport-Vassiliadis (RV) and Tetrathionate (TT) broths in parallel and incubated for an additional 24 h to reduce the other competitive bacteria present in the samples and allow only *Salmonella* to proliferate within these broths (Teague and Clurman, 1916; Knox, 1945; Vassiliadis, 1983; Peterz et al., 1989). Followed by, the selectively enriched samples were streaked onto selective agar plates such as Xylose lysine Tergitol-4 (XLT-4) agar and incubated for another 24 h. After incubation, one to three presumptive *Salmonella* colonies are picked and restreaked before confirmation by various molecular, biochemical, or serological tests, including somatic (O) antigen agglutination tests and flagellar (H) antigen agglutination tests (Andrews et al., 2018). Thus, traditional *Salmonella* isolation at a minimum takes 4–5 days, and reducing this time may be beneficial.

BPW is a non-selective broth and this incubation is considered a non-selective pre-enrichment step that is required to resuscitate *Salmonellae*

Abbreviations: BPW, buffered peptone water; RV, Rappaport-Vassiliadis; TT, tetrathionate; CRISPR, clustered regularly interspaced short palindromic repeats; CRISPR-SeroSeq, serotyping by CRISPR sequences.

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from injury imparted during processing (D'Aoust, 1981; Busse, 1995). The selective chemicals like bile salts, brilliant green dye and malachite green dyes are added to the selective enrichment broths to reduce the other competitive bacteria in *Salmonella* isolation (Oscar Teague and A. W. Clurman, 1916; Rappaport et al., 1956; Vassiliadis et al., 1978; Peterz et al., 1989; Daquigan et al., 2016). In enrichment broths such as RV, selectivity depends on low pH (5.2), high ionic strength (due to $MgCl_2$) and malachite green. The magnesium chloride creates hypertonic conditions that inhibit growth of *Proteus* spp. And *E. coli* by osmotic pressure, whereas the presence of malachite green inhibits coliforms (Rappaport et al., 1956; Vassiliadis et al., 1981; 1983; Peterz et al., 1989). The conditions such as high osmotic pressures, low pH levels, resistance to malachite green and survive at less demanding nutritional requirements were well tolerated by *Salmonella* and thus helps in its proliferation (Dwivedi et al., 2014). TT broth contains an iodine-iodide (I_2-KI) solution that induces tetrathionate production which suppresses coliforms and other microflora, providing a metabolic advantage to microorganisms such as *Salmonella* that produce tetrathionate reductase (Knox, 1945; D'Aoust, 1981; Moats, 1981; Winter et al., 2010). TT broth also contains bile salts that cause oxidative DNA damage and some TT broth formulations also contain brilliant green, which blocks sucrose or lactose fermentation (Bakker et al., 1992; Bernstein et al., 1999; Merritt and Donaldson, 2009). The lipopolysaccharide (LPS) in Gram negative bacteria limits permeability of these agents to the cytosol, thus protects them from the action of bile salts and brilliant green (Provenzano et al., 2000; Cremers et al., 2014). TT Hajna broth contains the bile salt sodium deoxycholate and also contains brilliant green (Hajna and Damon, 1956).

The most common selective and differential agar used for *Salmonella* isolation is XLT-4, which has largely replaced xylose lysine deoxycholate (XLD) agar (USDA, 2014). *Salmonella* colonies present as distinct black colonies on both XLT-4 and XLD agars due to H_2S production. The presence of H_2S indicators such as sodium thiosulfate and ferric ammonium citrate act as differential agents from other Enterobacteriaceae (Miller et al., 1991; Mallinson et al., 2000). H_2S-negative strains appears as pinkish-yellow colonies on XLT-4 (Miller et al., 1991) and thus second selective media such as brilliant green sulphate (BGS) and Hektoen enteric (HE) are suggested (Komastu and Restaino, 1981; Mallinson et al., 2000). The inclusion of tergitol 4 in XLT-4 inhibits all gram-positive bacteria and also inhibits the growth of *Proteus* spp. And *Pseudomonas* spp. (Miller et al., 1991; Tate et al., 1990; Mallinson et al., 2000).

Novobiocin is a bacteriostatic antibiotic that inhibits bacterial DNA synthesis. The LPS present on Gram negatives limits permeability of novobiocin, so this antibiotic typically targets Gram positive bacteria (Bisacchi and Manchester, 2015; May et al., 2018). Further LPS modifications in *Salmonella* render it more resistant to novobiocin than other gram negatives such as *Klebsiella* spp., *E. coli,* and *Proteus* spp. (Sabath et al., 1968; Brock, 1987; Nobre et al., 2015). Novobiocin is often added to BGS and HE agars and XLD to increase *Salmonella* selection (Jeffries, 1959; Hoben et al., 1973; Moats, 1978; Komastu and Restaino, 1981; Devenish et al., 1986; Gast and Porter, 2020).

Earlier studies have sought to increase selection at different stages in *Salmonella* isolation, chiefly to reduce the background microflora in highly contaminated samples. For example, by adding novobiocin to BPW or TT broths (Jeffries, 1959; Jensen et al., 2003; Wang and Hammack, 2014) or by adding malachite green to BPW broth (Schothorst and Renaud, 1985). *Salmonella enterica* is a hugely diverse species that is represented by over 2500 distinct serovars that are defined by their cell-surface O and H antigens (Grimont and Weill, 2007). Past studies indicated these serovars exhibit different growth characteristics even when grown in same enrichment, thus introducing biases when enriched (Harvey and Price, 1967; Singer et al., 2009; Temelli et al., 2010; Gorski, 2012; Cox et al., 2019a, 2019b, 2020). For example, serovars Heidelberg and Senftenberg were preferentially recovered from TT broth compared to Gram negative broth (Cox et al., 2020), serovar Newport showed dominance over serovar Typhimurium and serovar Enteritidis when grown together (Singer et al., 2009), serovar Enteritidis grew better in the RV enrichment broth variant, R-10 (Singer et al., 2009), and serovars Typhimurium and Saintpaul exhibit reduced growth in RV broth (Gorski, 2012). On plating media, serovar Kentucky appears to be more frequently isolated from XLT-4 than BGS (Cox et al., 2020). Some serovars cannot be isolated on XLT-4 media, including serovar Typhi and many strains of serovar Cholerasuis (Mallinson et al., 2000; Miller et al., 1991; Miller and Mallinson, 2000). Serovar Typhi cannot be propagated in RV broth, as it is sensitive to malachite green, so TT broth serves as the best enrichment when this serovar is present (Wang and Hammack, 2014).

Salmonella contains two CRISPR loci; CRISPR 1 and CRISPR 2, which are located <10 kb apart (Touchon and Rocha, 2010; Fabre et al., 2012). The direct repeats are 29 nucleotides long and spacers are 32 nucleotides long (Touchon and Rocha, 2010). CRISPR spacer content is highly conserved within a specific serovar, and this property can be used to effectively discern *Salmonella* serovars (Liu et al., 2011; Fabre et al., 2012; Shariat et al., 2013; Bachmann et al., 2014; Rauch et al., 2018; Vosik et al., 2018; Richards et al., 2020). CRISPR-SeroSeq is a molecular tool that utilizes next generation amplicon sequencing of *Salmonella* CRISPR arrays to identify multiple serovars in a single sample (Thompson et al., 2018). Using this approach, we recently reported that serovars Enteritidis and Schwarzengrund were preferentially enriched in TT and RV broths respectively (Cox et al., 2019a, 2019b).

We postulated that increasing the selectivity of the pre-enrichment step would not only reduce the background flora, as shown by others, but would enable direct plating from a selective pre-enrichment culture. This would avoid the need for an additional 24-h selective enrichment culture and reduce the overall time required for *Salmonella* isolation by a day. Our rationale in the current study was to provide sufficient suppression of background flora using these agents, such that *Salmonella* would recover and proliferate better with reduced competition in the pre-enrichment broth. Since the purpose of pre-enrichment is to allow resuscitation of damaged *Salmonellae*, the timing of adding these components is an important consideration, and this would be a delicate balance that requires combining non-selective and selective enrichment steps. One selective agent from RV broth (malachite green), one from TT broth (bile salts), and also novobiocin were selected for this study. Bile salts were chosen instead of tetrathionate as they have been broadly used to limit the growth of Gram-positive bacteria and because tetrathionate may be too selective. Our objective was to test whether the delayed addition of these selective agents during pre-enrichment step could reduce the time required to isolate *Salmonella*. Given the differences in *Salmonella* serovar isolation under different media conditions, we also used CRISPR-SeroSeq to investigate whether the selective pre-enrichments used here resulted in isolation of different serovars when compared to isolation after standard selective enrichment in RV and TT broths.

2. Materials and methods

2.1. Sample collection

Carcass drip samples were collected as described by Line et al. (2013). Briefly, a sanitized plastic tub was placed beneath the moving shackle line following feather removal in commercial poultry processing plants (Fig. 1). The tub remained in place collecting all dripping processing water for the amount of time needed to allow approximately 500 carcasses to pass as determined by counting carcasses with a timer. The time required varied depending on processing parameters and line speed; ranged from 2 min to 50 s to 4 min and 4 s. Carcass drip samples were transferred to sterile Nalgene bottles, which were sealed, placed on ice and shipped overnight in cold insulated packaging to the laboratory for bacterial culture. In Experiment 1, samples were collected in duplicate from four different processing plants for eight total samples and in

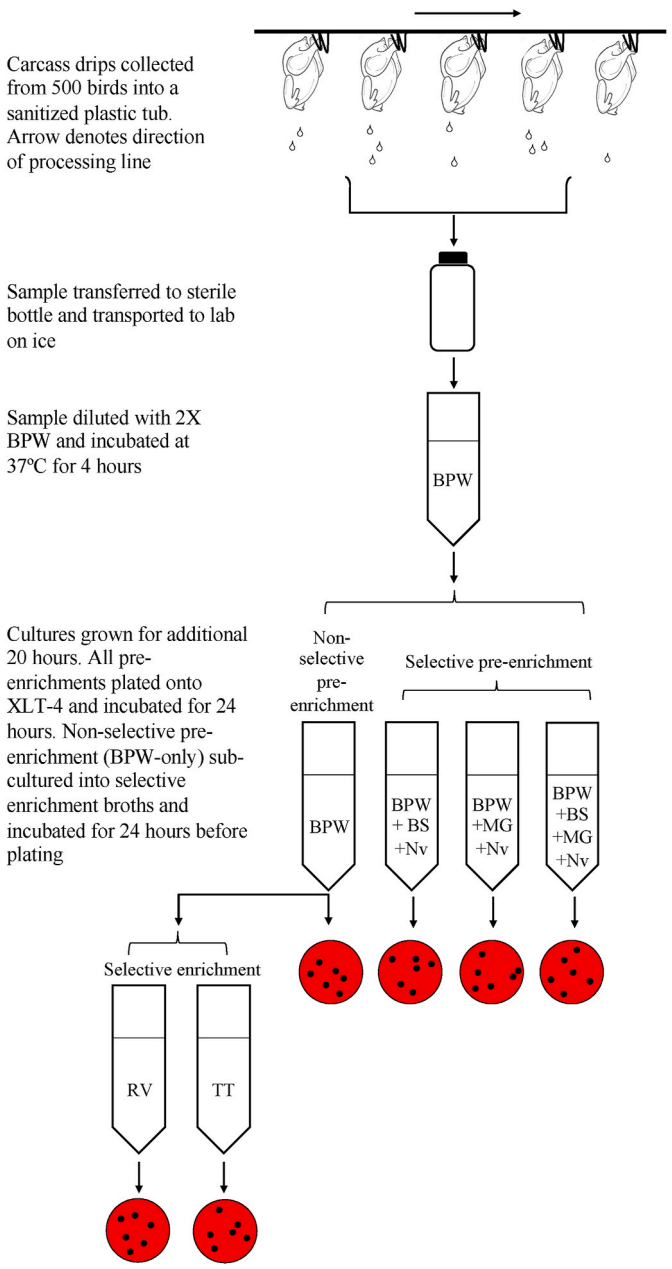

Fig. 1. Schematic of sample collection and culture conditions. Figure shows carcass drip line sample collection and workflow of *Salmonella* isolation by both conventional and experimental culture conditions. BPW, buffered peptone water; RV, Rappaport-Vassiliadis broth; TT, tetrathionate; BS, bile salts; Nv, novobiocin; XLT-4, Xylose Lysine Tergitol-4 agar.

Experiment 2, in duplicate from five different plants for ten total samples. For plants 2–4, and 5–9 dual processing lines were running, and a single drip line sample was collected from each line. From plant 1, duplicate samples were collected on the same line. In total 18 samples, representing nine different processing facilities, were examined in this whole study.

2.2. *Salmonella* isolation and confirmation

Samples were diluted 1:1 with double strength BPW (2XBPW) and incubated for 4 h at 37 °C in a shaking incubator (Fig. 1). Subsequently, four aliquots of 30 ml were placed into separate sterile culture flasks and selective ingredients were added in different combinations to the following final concentrations: novobiocin 0.015 g/L; bile Salts 1 g/L; malachite green 0.1 g/L (Hardy diagnostics, Santa Maria, CA). In Experiment 1, the following three combinations were used: i) bile salts and novobiocin, ii) malachite green and novobiocin, and iii) bile salts, malachite green, and novobiocin. In Experiment 2, the following three conditions were used: i) bile salts, ii) novobiocin, iii) bile salts and novobiocin. For all samples a non-selective BPW control was included. When BPW is used alone, the condition was referred to as non-selective pre-enrichment condition in the manuscript. The addition of malachite green, bile salts and novobiocin individually or in different combinations to the BPW were referred to as selective pre-enrichment condition in the manuscript. All culture flasks continued to incubate for an additional 20 h at 37 °C in a shaking incubator. After incubation, all broths were streaked for isolation onto XLT-4 agar (Hardy Diagnostics, Santa Maria, CA) and plates were incubated for 24 h at 37 °C (Fig. 1). The streaking of aliquots from either pre-enrichment or enriched cultures on XLT-4 agar was referred to as selective plating in the manuscript. As a positive control, 0.1 ml and 1 ml of the BPW-only non-selective pre-enrichment cultures were also sub-inoculated into RV and TT selective enrichment broths (Hardy Diagnostics, Santa Maria, CA) respectively (Fig. 1), performed according to conventional protocol followed by USDA-FSIS. The samples from non-selective BPW, when inoculated in to either RV or TT broth were referred to as selective enrichment condition in the manuscript. These selective enrichment broths were incubated for 24 h at 37 °C in a shaking incubator before being streaked onto XLT-4 agar (Fig. 1) which was incubated as described above. XLT-4 plates were examined for development of typical black H_2S colonies and up to six characteristic *Salmonella* colonies were picked and streaked for isolation onto new XLT-4 plates, which were incubated as described above. Isolated *Salmonella* colonies were serogrouped using *Salmonella* polyvalent and single factor antisera until mixture results in marked agglutination (Difco, Madison, WI) according to the manufacturer's instructions. The samples from Plant-9 were serogrouped by CRISR-SeroSeq, based on its relative frequency as major serovar within the sample.

2.3. DNA isolation and CRISPR-SeroSeq

One ml of each pre-enriched and enriched broth was centrifuged at 15,000 RPM for 2 min to pellet the bacteria, and the supernatant was removed. Pellets were stored at −20 °C until analysis. Total genomic DNA was isolated from samples found to be *Salmonella* positive by culture; isolation was done using the Genome Wizard Kit (Promega, Madison, WI) according to the manufacturer's instructions. DNA pellets were resuspended in 200 μL of molecular grade water and stored at −20 °C until use. DNA samples were diluted 1:20 in water and 2 μL was used as a template in the first step CRISPR-SeroSeq PCR to amplify *Salmonella* CRISPR spacer sequences with the following primers: forward primer, 5′- tcgtcggcagcgtcagatgtgtataagagacagcgcgccagcggggataaacc-3′ and reverse primer 5′- gtctcgtgggctcggagatgtgtataagagacaggctggcgcggggaa-cac-3' (Thompson et al., 2018). The underlined portion of the primers binds to the conserved direct repeat sequences found in. *Salmonella* CRISPR arrays, and result in a heterogeneous mix of PCR amplicons containing different spacers, typically 1–3 spacers per amplicon. A 5 μL aliquot of the PCR was analyzed by gel electrophoresis to confirm CRISPR amplification and the remaining 25 μL was purified using Ampure Beads (Beckman Coulter, Indianapolis, IN) according to manufacturer's protocol and resuspended in 40 μL of water. From this, 5 μL was used as a template in the second PCR with primers containing sequences for the addition of Illumina adaptors and dual barcodes as per the Illumina Nextera protocol (Illumina, San Diego, CA). Again, the PCR products were analyzed by gel electrophoresis and then were pooled in approximate equimolar proportions and sequenced using an Illumina NextSeq (Wright Labs, Huntingdon, PA). The pooled sequencing sample also contained two negative controls (water used as template in PCR1 and PCR2, and water used as a template for PCR2) and a single positive control (serovar Enteritidis genomic DNA with known CRISPR content).

Sequencing reads without a 100% dual-indexed barcode sequence match were removed. CRISPR-SeroSeq analyses were performed using a Python script that scans sequence reads and uses BLAST to match sequence reads to a database of over 130 serovars, before writing the output directly to Microsoft Excel (Thompson et al., 2018; Cox et al., 2019a, 2019b). For each plant sample, the reads for each sample were normalized to the reads from the culture condition with the lowest number of sequencing reads (Supplemental Table 1). From this normalization, individual spacer sequences with fewer than five reads were not included in the analysis. Serovars were confirmed only if they contained multiple CRISPR spacers that were unique to that serovar and if the cumulative number of reads for all spacers in that serovar constituted a relative frequency of at least 0.001% of the sample's total serovar population.

2.4. Statistical analysis

Fisher's exact test was used to compare the number of samples positive for *Salmonella* after enrichment in selective pre-enrichment media compared to non-selective media in all 18 samples and the *p* value was reported. A two-tailed *t*-test was used to compare mean serovar diversity between conventional enrichments and the selective pre-enrichments, with serovar diversity being determined by the number of different serovars detected in each sample under each condition.

3. Results

3.1. Experiment 1: Salmonella recovery in BPW with combinations of selective ingredients

Duplicate carcass drip samples were collected from four processing plants and the data are presented in Table 1. All eight samples were positive for *Salmonella* when pre-enriched in standard non-selective BPW and then selectively enriched in either RV (37.5%; 3/8 samples) or TT broth (87.5%; 7/8 samples). This includes both samples from Plant 2 that were positive after both RV and TT enrichment. All isolates from Plants 2–4 serogrouped as O:8, and most isolates from Plant 1 serogrouped as O:4 with one sample belongs to O:7 (Table 1). All eight samples were also positive after direct plating from at least one of the selective pre-enrichment conditions. Conversely, only 62.5% (5/8) of samples plated directly after non-selective pre-enrichment in BPW were positive for *Salmonella*. The addition of bile salts and novobiocin to BPW resulted in a more consistent recovery of *Salmonella* (75%; 6/8 samples) than in BPW alone and is concordant with *Salmonella* recovery from TT broth (Table 1). The addition of malachite green and novobiocin to BPW enabled early recovery of *Salmonella* from both Plant 1 samples and from a single sample from Plant 4. Notably, *Salmonella* from Plant 1 was not recovered using BPW with bile salts and novobiocin. Further, the addition of bile salts, malachite green, and novobiocin seemed to be over-selective, resulting in only four samples that were positive for *Salmonella* compared to BPW. In Experiment 1, we combined novobiocin with either bile salts or malachite green, rather than adding the components individually. Our rationale here was two-fold: i) to reduce the overall culture conditions to be tested, and ii) since RV and TT selection broths are used in parallel and each is often plated onto media containing novobiocin, combining them made sense. Since BPW containing bile salts and novobiocin yielded the greatest number *Salmonella* positive samples and had better recovery than any of the other selective pre-enrichment conditions, we sought to determine whether this required the individual action of the selective components, or the combination of both.

3.2. Experiment 2: Salmonella recovery in BPW with individual selective ingredients

In Experiment 2, carcass drip samples were collected in duplicate from an additional five different broiler processing plants (plants 5–9) and the resulting 10 samples were pre-enriched with either bile salts or novobiocin, or both together. As above, all samples were positive after pre-enriched in non-selective BPW, followed by selective enrichment in at least one of the broths either RV or TT. RV enrichment yielded 70% positive samples (7/10 samples) and TT enrichment resulted in 100% *Salmonella* positive samples (Table 2). Similar to Experiment 1, most isolates were grouped as serogroup O:8 (Table 2). Only 70% of samples yielded *Salmonella* colonies when plated directly after non-selective pre-enrichment in BPW (Table 2), and these were different from those found to be positive post RV enrichment. Selective pre-enrichment with bile salts alone or novobiocin alone yielded 90% and 80% samples that were *Salmonella* positive, respectively, whereas the combination of bile salts and novobiocin resulted in 100% *Salmonella* recovery (Table 2). Collectively, for plants 1–9, increasing the selectivity of the pre-enrichment step (with either bile salts and novobiocin or malachite green and novobiocin) enabled recovery of *Salmonella* from all samples compared to direct plating from a non-selective pre-enrichment with BPW-alone (Fisher's exact test, $p = 0.0191$).

3.3. Salmonella serovar profiles under different pre-enrichment and enrichment conditions

Since media choice can influence the growth of different serovars, we employed CRISPR-SeroSeq to assess serovar populations within individual samples. CRISPR-SeroSeq was performed on positive samples from Experiments 1 and 2 that were selectively pre-enriched with bile salts and novobiocin combined, and from samples selectively enriched in RV or TT broth, whichever yielded *Salmonella* colonies. We also examined samples from plant 1, where the selective pre-enrichment condition with the addition of malachite green and novobiocin yielded *Salmonella* colonies while other selective pre-enrichment conditions failed to recover *Salmonella*. We could not accurately analyze the samples shown positive from non-selective BPW due to low reads obtained, attributed to presence of other Enterobacteriaceae whose DNA can inhibit the PCR. We did not attempt performing CRISPR-SeroSeq on positives recovered with bile salts, malachite green and novobiocin combination in BPW due to its over selective nature, inconsistent

Table 1
Salmonella recovery after pre-enrichment in different selective conditions[a].

Culture condition[b]		Plant 1		Plant 2		Plant 3		Plant 4		Total no.of positives
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	
Non-selective pre-enrichment	BPW	O:4	–	O:8	O:8	–	O:8	–	O:8	5/8
Selective pre-enrichment	BPW + BS + Nv	–	–	O:8	O:8	O:8	O:8	O:8	O:8	6/8
Selective pre-enrichment	BPW + MG + Nv	O:4	O:4	–	–	–	–	–	O:8	3/8
Selective pre-enrichment	BPW + BS + MG + Nv	–	O:4	–	–	–	O:8	O:8	O:8	4/8
Selective enrichment	BPW into RV	–	O:7	O:8	O:8	–	–	–	–	3/8
Selective enrichment	BPW into TT	O:4	–	O:8	O:8	O:8	O:8	O:8	O:8	7/8

[a] Serogroups: O:4 (B), O:7 (C1), O:8 (C2-3), O:9 (D1).
[b] BPW, buffered peptone water; RV, Rappaport-Vassiliadis broth; TT, tetrathionate; BS, bile salts; Nv, novobiocin; MG, malachite green.

Table 2
Comparison of *Salmonella* recovery after pre-enrichment with bile salts and novobiocin, individually and in combination[a].

Culture condition[b]		Plant 5		Plant 6		Plant 7		Plant 8		Plant 9[c]		Total no.of positives
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	
Non-selective pre-enrichment	BPW	O:8	O:8	–	–	O:8	O:8	O:8	O:8	O:8	–	7/10
Selective pre-enrichment	BPW + BS + Nv	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	–	9/10
Selective pre-enrichment	BPW + MG + Nv	O:8	O:8	–	–	O:8	O:8	O:8	O:8	O:8	O:8	8/10
Selective pre-enrichment	BPW + BS + MG + Nv	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	10/10
Selective enrichment	BPW into RV	–	O:8	O:8	–	O:8	O:8	O:8	O:8	O:8	–	7/10
Selective enrichment	BPW into TT	O:7	O:8	O:8	O:9	O:8	O:8	O:4	O:7	O:8	O:4	10/10

[a] Serogroups: O:4 (B), O:7 (C1), O:8 (C2-3), O:9 (D1).
[b] BPW, buffered peptone water; RV, Rappaport-Vassiliadis broth; TT, tetrathionate; BS, bile salts; Nv, novobiocin.
[c] Serogroups for Plant 9 determined by CRISPR-SeroSeq.

recovery and to maintain a comparison between both Experiments 1 and 2.

When considering duplicates and different culture conditions as different individual samples, a total of 44 CRISPR-SeroSeq samples were present and there were about four to six samples per plant (plants 1–9). We identified nine different serovars and one untypeable serovar (Fig. 2). The average number of serovars detected per culture sample was two, and the most diverse samples contained four different serovars. Regarding serovar presence and their relative frequency data (Fig. 2), fourteen samples harbored a single serovar, and in all cases, that was serovar Kentucky. It was the most frequently (40/44 samples) identified and predominant serovar (37/40) found in all plants with only one exception, not detected from plant 1, replicate 2 (Fig. 2). This is concordant with the serogrouping data presented in Tables 1 and 2, as serovar Kentucky expresses the O:8 antigen. Serovar Typhimurium was detected in samples from three different plants (plants 1, 8, and 9), and interestingly for plants 8 and 9, it was a minority serovar (i.e., not the most abundant serovar) in all, but one sample (Fig. 2). As in previous work, we also saw a propensity for serovar Enteritidis enrichment in TT compared to RV (plant 5, replicate 2 and plant 9, replicate 1). Serovar Berta was seen only in samples from plant-7 in both selectively pre-enriched and enriched culture conditions. Serovar Braenderup was found in five samples, and four of these were selectively enriched cultures, which suggests that this serovar is not as competitive in the selective pre-enrichment environment as in the enrichments. Serovars Mbandaka, Infantis and Montevideo were each only identified in single culture samples, when culture conditions consist of bile salts in either selective pre-enriched or enriched cultures.

Occasionally, the relative abundance between the serovars was different, depending on the culture condition used, such as the switch between serovars Typhimurium and Schwarzengrund in replicate 2 from plant 1, or between serovars Typhimurium and Kentucky in replicate 2 from plant 9 (Fig. 2). There were 4/18 instances where the predominant serovar found after selective enrichment in at least one of RV or TT was not the predominant serovar after selective pre-enrichment. In these cases, the serovar was also identified in the pre-enriched sample, albeit at a lower relative frequency. The mean number of serovars detected in selectively enriched samples (RV or TT broth) compared to selective pre-enriched samples was not altered much with any significant statistical difference (two-tailed *t*-test, $p = 0.62$), suggesting no change in serovar diversity. Overall, the profile of serovars found in selectively enriched cultures was comparable to those found in selective pre-enriched cultures (Fig. 2).

4. Discussion

Salmonella outbreaks are often linked to poultry and poultry-related products; between 2010 and 2018, 87 multistate outbreaks were associated with poultry, resulting in more than 6200 cases of salmonellosis (Centers for Disease Control and Prevention, 2020). Conventional culture-based methods used by USDA-FSIS and the US Food and Drug Administration to isolate *Salmonella* involve protocols that take multiple days to complete (Andrews et al., 2018; USDA, 2014). While there are commercial products available that enable rapid determination of whether a sample is *Salmonella* positive (e.g., *Salmonella* BAX system, SureTect from ThermoFisher, and DNable *Salmonella* detection from

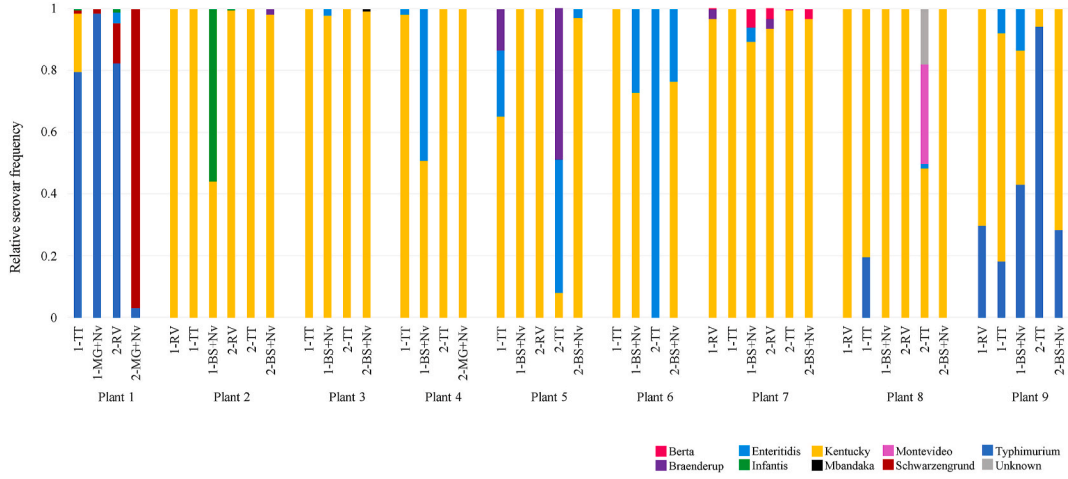

Fig. 2. *Salmonella* serovar identities in carcass drip samples cultured under different pre-enrichment and enrichment conditions. Aliquots of selective pre-enrichment (BPW plus additives) and enrichment (RV or TT) cultures that were *Salmonella* positive were analyzed by CRISPR-SeroSeq. The graph shows the relative frequency of each serovar within individual samples, down to 0.001% of the population. Each serovar is represented by a different color, as indicated. BPW, buffered peptone water; RV, Rappaport-Vassiliadis broth; TT, tetrathionate; BS, bile salts; Nv, novobiocin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

EnviroLogix), there is still a need to be able to isolate and characterize individual *Salmonella* colonies, especially within the context of an outbreak. Our findings here demonstrate that addition of ingredients that confer *Salmonella* selection in RV or TT enrichment broths can be added to the pre-enrichment step and allow for *Salmonella* isolation recovery a day earlier.

Early studies showed that a non-selective pre-enrichment step is required before selection in RV or TT broths for the successful recovery of *Salmonella* isolates in both artificial and naturally contaminated samples (Edel and Kampelmacher, 1973; D'Aoust, 1981; Hoorfar and Visby Mortensen, 2000). Many studies have addressed reducing the time required for *Salmonella* isolation, either by shortening the pre-enrichment step or by removing it entirely. There have been discrepancies in the time required for pre-enrichment, with one paper reporting that five to 6 h is sufficient, while another showed that 24 h was required (D'Aoust et al., 1990; Chen et al., 1993; Daquigan et al., 2016). Shortening the pre-enrichment time often resulted in false negatives (D'Aoust et al., 1990; Moats, 1981). Two studies showed higher *Salmonella* recovery rates from poultry farm samples when directly enriched in either selenite cystine or TT broth without pre-enrichment (Pangloli et al., 2003; Schultz et al., 2012). In the Schultz study, TT broth served as a pre-enrichment for enrichment in modified semi-solid RV agar (Schultz et al., 2012), and this approach has been approved by the National Poultry Improvement Plan for environmental samples collected during live production.

Other groups have investigated the addition of selective ingredients to BPW, including novobiocin and malachite green (individually), and reported increase in *Salmonella* recovery (Jensen et al., 2003; Schothorst and Renaud, 1985) and also the addition of novobiocin or sodium deoxycholate (the selective component of bile salts) in improving Shiga toxin-producing *E. coli* isolation (Amagliani et al., 2018; Margot et al., 2015). However, we know of no previously published work on investigating selective pre-enrichment for *Salmonella* recovery in poultry processing samples by comparing combinations of bile salts, malachite green and novobiocin being added to BPW.

To allow the resuscitation of potentially injured *Salmonella* and enable multiplication during the lag phase, we pre-enriched the samples in non-selective BPW for 4 h before introducing any selection. We did not find any false negatives as all samples were positive after addition of bile salts and novobiocin or malachite green and novobiocin to the pre-enrichment media, with the former combination resulting in recovery from 89% (16/18) samples. While separate addition of bile salts or novobiocin resulted in high *Salmonella* recovery, the combination of both showed slightly better recovery, which is likely due to the combinatorial action of novobiocin and bile salts in inhibiting gram positives, and also *Proteus* spp. It is necessary to maintain a balance between a selective pressure that limits growth of competitive bacteria and an ability for injured *Salmonella* to recover (Carrique-Mas and Davies, 2008). Our data supports this, where addition of all three components together (bile salts, malachite green, and novobiocin) to BPW resulted in lower *Salmonella* recovery rates among all selective pre-enrichment conditions investigated, likely due to excessive selective pressure. In this work, we used the selective ingredients at the same concentration that they are used at in selective enrichment broths (bile salts, 1 g/L, and malachite green, 0.1 g/L). The culture-fitness characteristics can change based upon the concentration of ingredients used and pH of the pre-enrichment media (Gorski, 2012), therefore future work will include titrating the concentrations of each component to determine whether more optimal concentrations can be used to improve *Salmonella* isolation.

Salmonella isolation from plant 1 samples were unique across the nine plants we investigated. Here, addition of malachite green and novobiocin to the pre-enrichment allowed early recovery, whereas no *Salmonella* was recovered from the bile salts and novobiocin culture. This sample contained high relative amounts of serovar Schwarzengrund. Previous work from our group has demonstrated that this serovar is isolated at higher frequency after enrichment in RV compared to TT (Cox et al., 2019a, 2019b), therefore it is not surprising that we were able to recover it in malachite green, a component of RV.

Previous studies have shown that different *Salmonella* serovars are recovered from the same samples when enriched in different selective enrichment broths (Gorski, 2012; Cox et al., 2019a, 2019b). Further, it is becoming more evident that *Salmonella* exists in multi-serovar populations in broiler houses, and also on individual carcasses at processing (Berghaus et al., 2013; Cox et al., 2019a, 2019b; Thompson et al., 2018) and that picking and characterizing only a small number of colonies from indicator agar only represents the most abundant serovars present in a sample (Cox et al., 2019a, 2019b). Therefore, using CRISPR-SeroSeq, we performed an in-depth analysis on the culture-positive *Salmonella* samples to investigate the profiles of serovars found between selective pre-enrichment broth with bile salts and novobiocin and selective enrichment broth.

Across the dataset, the identity of serovars found within samples from the same plant matched, with few exceptions. With the exception of Braenderup in plant 5-replicate 2, the predominant serovar in all enriched samples, that with the highest relative frequency within a sample, was also detected in the selective pre-enriched samples. While there was no statistical difference in the level of serovar diversity between enriched and selectively pre-enriched samples, we noted that in 39% (7/18) cases, the number of serovars was higher in enriched samples, compared to 28% cases where that number was higher in selectively pre-enriched samples, and the remaining 33% samples where the number of serovars matched between the two treatments.

There are caveats to the work presented here, namely that we collected our samples post-pick, but before the carcasses were exposed to many of the antimicrobial processing aids which may be used during broiler processing. This was done deliberately as we anticipated that these samples would be more likely to be *Salmonella* positive and would therefore allow us to test a larger number of samples from different plants in the context of this study. Further, the use of carcass drip samples enabled us to assess *Salmonella* in a larger number of birds, which is more reflective of the flock than a smaller number of carcasses (Line et al., 2013). It is possible that a higher *Salmonella* load at this location during processing enabled us to isolate *Salmonella* using the selective pre-enrichment conditions, and that at later points in processing (i.e., post chilling) where the quantity of *Salmonella* is reduced, that our methodology may yield false negatives. Nonetheless, for samples that may have a high *Salmonella* load (e.g., chicken cecal samples, or environmental samples collected from broiler or breeder houses), this methodology reduces the time and cost burden for *Salmonella* isolation. Future work will involve testing individual carcasses collected post chilling and will compare carcass drip sampling directly to individual carcass rinses. Future work would also compare a selective pre-enrichment with established *Salmonella* isolation protocols from carcass rinses. The concentrations of each additive to BPW were matched to the concentration used in its native media; future studies may reveal optimal concentrations. Future work would also involve plating on multiple selective media to reduce any incidence of false negatives. In this work, all 18 plant samples were positive and since serovar population analyses was performed on multiple sub-samples from each collection, we do not expect that we missed any false negatives in this work. From a technical perspective, the 4-h incubation might be challenging for technical labs who receive samples late in the day. The required concentration of selective components for successful inhibition of background flora and the resulting *Salmonella* recovery rate may change based upon the complexity and matrix of the samples.

5. Conclusions

In conclusion, our results suggest that addition of bile salts and novobiocin to BPW provides sufficient selection to enable *Salmonella* recovery 24 h earlier than afforded by conventional *Salmonella* isolation

that requires a pre-enrichment step followed by selective enrichment. Importantly, the serovar profiles do not alter dramatically between pre-enrichment and enrichment, for the majority of samples studied here. Though performed in poultry, the work presented here is relevant to multiple different food industries where *Salmonella* surveillance is routinely performed.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103818.

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